

Selection, characterization and application of new RNA HIV gp 120 aptamers for facile delivery of Dicer substrate siRNAs into HIV infected cells

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ABSTRACT

The envelope glycoprotein of human immunodeficiency virus (HIV) consists of an exterior glycoprotein (gp120) and a *trans*-membrane domain (gp41) and has an important role in viral entry into cells. HIV-1 entry has been validated as a clinically relevant anti-viral strategy for drug discovery. In the present work, several 2'-F substituted RNA aptamers that bind to the HIV-1_{BaL} gp120 protein with nanomole affinity were isolated from a RNA library by the SELEX (Systematic Evolution of Ligands by EXponential enrichment) procedure. From two of these aptamers we created a series of new dual inhibitory function anti-gp120 aptamer-siRNA chimeras. The aptamers and aptamer-siRNA chimeras specifically bind to and are internalized into cells expressing HIV gp160. The Dicer-substrate siRNA delivered by the aptamers is functionally processed by Dicer, resulting in specific inhibition of HIV-1 replication and infectivity in cultured CEM T-cells and primary blood mononuclear cells (PBMCs). Moreover, we have introduced a 'sticky' sequence onto a chemically synthesized aptamer which facilitates attachment of the Dicer substrate siRNAs for potential multiplexing. Our results provide a set of novel inhibitory agents for blocking HIV replication and further validate the use of aptamers for delivery of Dicer substrate siRNAs.

INTRODUCTION

The global epidemic of infection by HIV has created an urgent need for new classes of antiretroviral agents.

HIV-1 infection is initiated by the interactions between the external envelope glycoprotein gp120 of HIV and the human cell surface receptor CD4 (1–4), subsequently leading to fusion of the viral membrane with the target cell membrane (5,6). Thus, HIV-1 entry into its target cells represents an attractive target for new anti-HIV-1 drug development (7–12).

The most effective anti-HIV drugs used in the clinic consist of combinations of compounds that block the early reverse transcription step, and the late viral gag protein maturation step (13–15). Preventing the virus from binding to its primary receptor is one of the most obvious and direct ways to prevent infection. One rational antiviral approach is to create some small ligands directed to the virus surface protein gp120. Successes in discovering new classes of gp120-directed inhibitors targeted to the initial step of HIV-1 entry have been reported in recent years (8,16,17). Soluble CD4 (sCD4) has demonstrated efficacy against many laboratory strains (18,19), however disappointing results from clinical trials were attributed to poor activity of this drug against primary isolates (20,21). Another entry inhibitor PRO-542, a CD4-IgG recombinant fusion protein effectively neutralizes many HIV-1 strains in cell culture (22,23) as well as clinical isolates (24,25), but unfortunately this drug has not enjoyed widespread use in the clinical treatment of HIV infection. Other inhibitors bind gp120 less specifically through electrostatic or lectin-carbohydrate interactions [sulfated polymers, cyanovirin (26)]. These properties create challenges for the practical implementation of these molecules as drugs. In 2003, Martin *et al.* (27) designed a 27-aa CD4 peptide mimic, CD4M33, which was shown to bind to gp120 and inhibit HIV-1 infection *in vitro*. The clinical application of this peptide though has not yet taken place. Aside from antibody or peptide mimics, nucleic acid aptamers with low to mid-range nanomole binding

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affinities are an attractive class of therapeutic molecules (27–30). Aptamers which block the interaction of gp120 and CD4 have been previously developed. One such aptamer was shown to specifically interact with the conserved coreceptor interacting region of R5 strain gp120 (29).

Although both aptamers and antibody or engineered peptide mimics have great specificity and binding affinity, the nucleic acid-based aptamers offer more synthetic accessibility, convenient modification, chemical versatility, stability and lack immunogenicity (31). Therefore, aptamers can be utilized for flexible applications ranging from diagnostic to therapeutic assay formats (32,33). Aptamers that target specific cell surface proteins are employed as interesting delivery molecules to target a distinct cell type, hence reducing off-target effects or other unwanted side effects. The first example of aptamers used for siRNA delivery is an anti-PSMA aptamer (34) that binds tightly to the prostate-specific membrane antigen (PSMA), a prostate cancer cell-surface receptor. McNamara *et al.* (35) in 2006 reported the use of this aptamer for receptor mediated siRNA delivery. In this study, they selectively delivered a 21-mer siRNA into PSMA expressing cells, resulting in silencing of target transcripts both in cell culture and *in vivo* following intra-tumoral delivery. Similarly, Chu *et al.* (36) used a modular streptavidin bridge to connect lamin A/C or GAPDH siRNAs to the biotinylated variants of this anti-PSMA aptamer. Consequently, this system induced silencing of the targeted genes only in cells expressing the PSMA receptor. The targeting properties of the anti-PSMA aptamer also can be exploited for localizing other therapeutic agents to tumors such as a toxin (37), doxorubicin (38) or nanoparticles (39–42). We recently described a novel dual inhibitory function anti-gp120 aptamer-siRNA chimera, in which both the aptamer and the siRNA portions have potent anti-HIV activities (43). Additionally, HIV gp120 expressed on the surface of HIV infected cells was used for aptamer-mediated delivery of an anti-HIV siRNA, resulting in pronounced inhibition of HIV replication in cell culture.

For treatment of HIV using aptamer-siRNA chimeras, it is highly desirable to generate new aptamers to expand the diversity of target recognition for potential use *in vivo*. New anti-gp120 aptamers and various siRNAs targeting different genes could be combined to avert viral resistance to a single aptamer-siRNA combination. In the present study we successfully isolated several new 2'-F substituted RNA aptamers that bind to the HIV-1_{BaL} gp120 protein. Two different types of dual inhibitory function anti-gp120 aptamer-siRNA conjugates were constructed. One of these is a covalent aptamer-siRNA chimera and the other is an aptamer with a GC-rich bridge that facilitates the interchange of different RNAs with the same aptamer. Although the aptamer alone provided HIV inhibitory function, the aptamer-siRNA chimeras provided more potent inhibition of HIV suggesting cooperativity between the siRNA and

aptamer portions in inhibiting HIV replication and spread. Our results demonstrate that our selected aptamers not only provide lead inhibitors for potential anti-HIV therapeutic applications, but also act as effective delivery vehicles for anti-HIV siRNAs into HIV infected cells. The aptamers studies here could also selectively deliver other small HIV inhibitory agents.

MATERIALS AND METHODS

Materials

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich, all restriction enzymes were obtained from New England BioLabs (NEB) and all cell culture products were purchased from GIBCO (Gibco BRL/Life Technologies, a division of Invitrogen). Sources for the other reagents were: DuraScribe T7 transcription Kit (EPICENTRE Biotechnologies); Silencer siRNA Labeling Kit (Ambion); Hoechst 33342 (nuclear dye for live cells) (Molecular Probes, Invitrogen); Random primers (Invitrogen); Bio-Spin 30 Columns (Bio-Rad); Recombinant Human Dicer Enzyme Kit (Ambion); Lipofectamine 2000 (Invitrogen); CHO-Env Transfectants (CHO-WT and CHO-EE), the HIV-1_{BaL} gp120 protein and HIV-1_{BaL} virus were obtained from the AIDS Research and Reference Reagent Program.

siRNAs

siRNAs and antisense strand RNAs were purchased from Integrated DNA Technologies (IDT).

Generation of aptamer and chimera RNAs by *in vitro* transcription

Aptamer and chimera RNAs were prepared as previously described (43). The sense strands of the chimeras are underlined. The italic *UU* is the linker between the aptamer and siRNA portions.

A-1 aptamer: 5'-GGGAGGACGAUGCGGAAUUGAG
GGACCACGCGCUGCUUGUUGUGAUAAAGC
AGUUUGUCGUGAUGGCAGACGACUCGCC
CGA-3'

B-68 aptamer: 5'-GGGAGGACGAUGCGGACAUAUAG
UAAUGACACGGAGGAUGGAGAAAAACA
GCCAUCUCUUGACGGUCAGACGACUCGCC
CGA-3'

Chimera A-1-sense strand: 5'-GGGAGGACGAUGCGG
AAUUGAGGGACCACGCGCUGCUUGUUGU
GAUAAGCAGUUUGUCGUGAUGGCAGACG
ACUCGCCCGA *UU* GCGGAGACAGCGACGA
AGAGCUCAUCA-3'

Chimera B-68-sense strand: 5'-GGGAGGACGAUGCG
GACAUAUAGAAUGACACGGAGGAUGGAGA
AAAAACAGCCAUCUCUUGACGGUCAGACG
ACUCGCCCGA *UU* GCGGAGACAGCGACGA
AGAGCUCAUCA-3'

Antisense strand: 5'-UGAUGAGCUCUUCGUCGCUG
UCUCCGCdTdT-3'

Aptamer-Stick-siRNA

A-1-stick, Stick-sense and stick-antisense were chemically synthesized by the Synthetic and Biopolymer Chemistry Core in the City of Hope (the details shown in the Supplementary Material). The A-1-stick RNA was refolded in HBS buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 2.7 mM KCl), heated to 95°C for 3 min and then slowly cooled to 37°C. The incubation was continued at 37°C for 10 min. The Sense-stick or Antisense-stick strand was annealed to the complementary partner using the same molar amounts as the corresponding partner strand to form the stick-siRNAs (NS-1 and NS-2). The same amount of the refolded A-1-stick was added and incubated at 37°C for 10 min in HBS buffer to form the A-1-stick-siRNA (N-1 and N-2).

A-1-stick: 5'-GGGAGGACGAUGCGGAAUUGAGGG
ACCACGCGCUGCUUGUUGUGAAUAGCAGU
UUGUCGUGAUGGCAGACGACUCGCCCGA
XXXXXXXX GUACAUCUAGAUAGCC-3'

Tat/rev N-1:

Antisense-Stick: 5'-UGAUGAGCUCUUCGUCGCUG
UCUCCGC *XXXXX* GGCUAUCUAGAAUGUA
C-3'

Sense strand: 5'-GCGGAGACAGCGACGAAGAGCUC
AUCAUU-3'

Tat/rev N-2:

Stick-sense: 5'-GCGGAGACAGCGACGAAGAGCUC
AUCA *XXXXX* GGCUAUCUAGAAUGUAC-3'

Antisense strand: 5'-UGAUGAGCUCUUCGUCGCUG
UCUCCGCUU-3'

TNPO3N-1:

Antisense-stick: 5'-UACACGAGCUGCAAUGUCGG
CUUUGCU *XXXXX* GGCUAUCUAGAAUGUA
C-3'

Sense strand: 5'-CAAAGCCGACAUUGCAGCUCGUG
UAUU-3'

CD4N-2:

Stick-sense: 5'-UCAAGAGACUCCUCAGUGAGAAG
AA *XXXXX* GGCUAUCUAGAAUGUAC-3'

Antisense strand: 5'-UUCUUCUCACUGAGGAGUCU
CUUGAUU-3'

The bold nucleotides indicate 2'-Fluorine modified sugars. The stick portion is underlined. This contains 2'-OMe modified A and G and 2'-F modified U and C. The italic *X* indicates the three-carbon linker (C3) between the aptamer/siRNA and stick sequences.

Preparation of the RNA library

The starting DNA library contained 50 nucleotides of random sequences and was synthesized by Integrated DNA Technologies (Coralville, Iowa). The random region is flanked by constant regions, which include

the T7 promoter for *in vitro* transcription and a 3' tag for RT-PCR. The 5' and 3' constant sequences are 5'-TAA TAC GAC TCA CTA TAG GGA GGA CGA TGC GG-3' (32-mer) and 5'-TCG GGC GAG TCG TCT G-3' (16-mer), respectively. The DNA random library (0.4 μM) was amplified by PCR using 3 μM each of 5'- and 3'-primers, along with 2 mM MgCl₂ and 200 μM of each dNTP. In order to preserve the abundance of the original DNA library, PCR was limited to 10 cycles. After the PCR reactions (10 reactions, 100 μl per reaction), the amplified dsDNA pool was recovered using a QIAquick Gel purification Kit. The resulting dsDNA was converted to an RNA library using the DuraScript Kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions. In the transcription reaction mixture, CTP and UTP were replaced with 2'-F-CTP and 2'-F-UTP to produce ribonuclease resistant RNA. The reactions were incubated at 37°C for 6 h, and subsequently the template DNA was removed by DNase I digestion. The transcribed RNA pool was purified in an 8% polyacrylamide/7M urea gel. The purified RNA library was quantified by UV spectrophotometry.

In vitro selection of RNA aptamers

The SELEX was performed principally as described by Tuerk and Gold (44). In every round, the RNA pools were refolded in HBS buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 2.7 mM KCl), heated to 95°C for 3 min and then slowly cooled to 37°C. Incubation was continued at 37°C for 10 min.

Generally, in order to minimize nonspecific binding with the nitrocellulose filters, the refolded RNA pools were preadsorbed to a nitrocellulose filter (HAWP filter, 0.45 μm) for 30 min, prior to incubation with the HIV-1_{Bal} gp120 protein. The precleared RNA pool was incubated with the target protein in low-salt RNA binding buffer (10 mM HEPES pH 7.4, 50 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 2.7 mM KCl, 10 mM DTT, 0.01% BSA and tRNA) for 30 min for SELEX rounds 1 to 4. After the fourth round of SELEX, a high-salt RNA binding buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 2.7 mM KCl, 10 mM DTT, 0.01% BSA and tRNA) was used. With the SELEX progress, the amount of gp120 protein was reduced and competitor tRNA was increased in order to increase the stringency of aptamer selection.

For the first cycle of selection, the precleared random RNA pool (40 μg, 1.5 nmol, 9 × 10¹⁴ molecules) and HIV-1_{Bal} gp120 protein (0.23 nmol, RNA/Protein ratio 6.5/1) were incubated in 200 μl low-salt RNA binding buffer on a rotating platform at room temperature for 30 min. The reaction was passed through a prewetted nitrocellulose filter and washed with 1 ml binding buffer. The bound RNA was eluted from the filter with 200 μl elution buffer (7M urea and 5 mM EDTA) at 95°C for 5 min, followed by phenol/chloroform extraction and concentration with a Microcon YM-30 column.

The recovered RNA pool was reverse transcribed using the ThermoScript RT-PCR system (Invitrogen) and amplified for 15 cycles of PCR. After the amplified dsDNA pool was purified using a QIAquick Gel purification Kit, it was transcribed as described above for the next round of selection.

After 12 rounds of SELEX, the resulting cDNA was amplified by PCR cloned into the TA cloning vector pCR 2.1 (Invitrogen). Individual clones were identified by DNA sequencing.

Gel shift assays and determination of dissociation constants

The gp120 protein was serially diluted to the desired concentrations (0–640 nM). A constant amount of P³²-end-labeled RNA (10 nM) was used. The binding reaction was performed as described above. After incubation, 20 µl of binding reaction was loaded into a 5% nondenaturing polyacrylamide gel. Following electrophoresis the gel was exposed to a Phosphor image screen and the radioactivity was quantified using a Typhoon scanner. The dissociation constants were calculated using non-linear curve regression with a Graph Pad Prism.

Cell culture

HEK 293 cells and CEM cells were purchased from ATCC and cultured in DMEM and RPMI 1640 supplemented with 10% FBS. CHO-WT and CHO-EE cells were obtained through the AIDS Research and Reference Reagent Program and were grown in GMEM-S. Cells were cultured in a humidified 5% CO₂ incubator at 37°C.

PBMCs. Peripheral blood mononuclear samples were obtained from healthy donors from the City of Hope National Medical Center. PBMCs were isolated from whole blood by centrifugation through a Ficoll-Hypaque solution (Histopaque-1077, Sigma). CD8 cells (T-cytotoxic/suppressor cells) were depleted from the PBMCs by CD8 Dynabeads (Invitrogen, CA) according to the manufacturer's instructions. CD8⁺ T cell-depleted PBMCs were washed twice in PBS and resuspended in culture media (RPMI 1640 with 10% FBS, 1 (PenStrep and 100 U/ml interleukin-2). Cells were cultured in a humidified 5% CO₂ incubator at 37°C.

Cell-surface binding of experimental RNAs (Flow cytometry analysis)

CHO-WT gp160 or CHO-EE cells were washed with PBS, trypsinized and detached from the plates. After washing cells twice with 500 µl binding buffer. Cell pellets were resuspended in binding buffer and incubated at 37°C for 30 min. Cells were then pelleted and resuspended in 50 µl of prewarmed binding buffer containing 400 nM Cy3-labeled experimental RNAs. After incubation at 37°C for 40 min, cells were washed three times with 500 µl of prewarmed binding buffer, and finally resuspended in 350 µl of binding buffer prewarmed to 37°C and analyzed by flow cytometry. For the aptamer-stick-siRNA

(N-1 and N-2), the Cy3-labeled aptamer-stick was incubated with the siRNA-stick to form the aptamer-stick-siRNA complex and the complexes were analyzed by flow cytometry.

Internalization and intracellular localization studies (Live-cell Confocal Microscopy analyses)

The CHO-WT gp160 and CHO-EE cells were grown in 35 mm plate (Glass Bottom Dish, MatTek, Ashland, MA, USA) with seeding at 0.3×10^6 in GMEM-S medium to allow about 70% confluence in 24 h. On the day of the experiments, cells were washed with 1 ml of prewarmed PBS, and incubated with 1 ml of prewarmed complete growth medium for 30 min at 37°C.

Assay 1. The cells were first stained by treatment with 0.15 mg/ml Hoechst 33342 (nuclear dye for live cells, Molecular Probes, Invitrogen, CA, USA) according to the manufacturer's instructions. Subsequently, Cy3-labeled RNAs at a 100 nM final concentration were added into the media and incubated for live-cell confocal microscopy in a 5% CO₂ microscopy incubator at 37°C. The images were collected every 15 min using a Zeiss LSM 510 Meta Inverted 2 photon confocal microscope system under water immersion at 40× magnification.

Assay 2. Cy3-labeled RNAs at a 100 nM final concentration were added to media and incubated for live-cell confocal microscopy in a 5% CO₂ microscopy incubator at 37°C. The images were collected every 15 min using a Zeiss LSM 510 Meta Inverted 2 photon confocal microscopy system under water immersion at 40× magnification. After 16 h of incubation and imaging, the cells were stained by treatment with 0.15 mg/ml Hoechst 33342 (nuclear dye for live cells, Molecular Probes, Invitrogen, CA, USA) according to the manufacturer's instructions. The images were collected as described previously.

HIV-1 challenge and p24 antigen assays

CEM cells or human PBMCs were infected with HIV III B or NL4-3 or Bal for 5 days (MOI 0.001 or 0.005). Prior to RNA treatments the infected cells were gently washed with PBS three times to remove free virus. Next, 2×10^4 infected cells and 3×10^4 uninfected cells were incubated with refolded RNAs at 400 nM final concentration in 96-well plates at 37°C. The culture supernatants were collected at different times (3 d, 5 d, 7 d and 9 d or 11 d). The p24 antigen analyses were performed using a Coulter HIV-1 p24 Antigen Assay (Beckman Coulter) according to the manufacturer's instructions.

qRT-PCR analysis

As previously described, the CEM cells or human PBMCs were infected with virus for 5 days. Prior to analyses, the infected cells were gently washed 3 times to eliminate free virus. The infected cells were treated directly with the experimental RNA (400 nM). After 7 days of incubation, total RNAs were isolated with STAT-60 (TEL-TEST

'B', Friendswood, TX, USA). Expression of the *tat/rev* coding RNAs was analyzed by quantitative RT-PCR using 2 (iQ SyberGreen Mastermix (BIO-RAD) and specific primer sets at a final concentration of 400 nM. Primers were as follows: IIIB and NL4-3 *tat/rev* forward primer: 5'-GGC GTT ACT CGA CAG AGG AG-3'; IIIB and NL4-3 *tat/rev* reverse primer: 5'-TGC TTT GAT AGA GAA GCT TGA TG-3'; Bal *tat/rev* forward primer: 5'-GAA GCA TCC AGG AAG TCA GC-3'; Bal *tat/rev* Reverse primer: 5'-TGC TTT GAT AGA GAA ACT TGA TGA-3'; CD4 forward primer: 5'-GCT GGA ATC CAA CAT CAA GG-3'; CD4 reverse primer: 5'-CTT CTG AAA CCG GTG AGG AC-3'. TNPO3 Forward primer: 5'-CCT GGA AGG GAT GTG TGC-3'; TNPO3 Reverse primer: 5'-AAA AAG GCA AAG AAG TCA CAT CA-3'; GAPDH forward primer 1: 5'-CAT TGA CCT CAA CTA CAT G-3'; GAPDH reverse primer 2: 5'-TCT CCA TGG TGG TGA AGA C-3'.

RNA-Stat60 was used to extract total RNA according to the manufacturer's instructions (Tel-Test). Residual DNA was digested using the DNA-free kit per the manufacturer's instructions (Ambion, CA, USA). cDNA was produced using 2 µg of total RNA Moloney murine leukemia virus reverse transcriptase and random primers in a 15 µl reaction according to the manufacturer's instructions (Invitrogen). GAPDH expression was used for normalization of the qPCR data.

In vitro Dicer assays

The sense or antisense strands were end-labeled with T4 polynucleotide kinase and γ -³²P-ATP. Subsequently, corresponding antisense or sense strands were annealed with equimolar amounts of 5'-end-labeled sense or antisense strands in HBS buffer to form the chimeras. The annealed stick-siRNA was incubated with refolded A-1-stick at 37°C for 10 min. The experimental RNAs (1 pmol) were incubated at 37°C for 40 min in the presence or in the absence of 1 U of human recombinant Dicer enzyme following the manufacturer's recommendations (Ambion, Austin, TX, USA). Reactions were stopped by phenol/chloroform extraction and the resulting solutions were electrophoresed in a denaturing 20% polyacrylamide gel. The gels were subsequently exposed to X-ray film.

RESULTS

Selection and identification of RNA aptamers against HIV-1_{BaL} gp120

An *in vitro* SELEX procedure (44–47) was used to select 2'-fluoropyrimidine modified RNA aptamers which selectively bind the R5 strain HIV-1_{BaL} gp120 envelope protein. To carry out the SELEX an RNA library containing a central stretch of 50 random nucleotides was synthesized by *in vitro* T7 transcription. Prior to selection for binding to the gp120 protein, the initial randomized RNA pool, which contained 10¹⁵ unique RNA

molecules (1.5 nmol), was preadsorbed to a nitrocellulose membrane to remove nonspecific binders. The pre-cleared RNA pool was then incubated with 0.23 nmol of HIV-1_{BaL} gp120 protein at room temperature. Subsequently, the RNA/protein complexes were isolated from the unbound RNA molecules *via* binding to a nitrocellulose filter. The selected RNAs were recovered from the filter, reverse transcribed and the cDNA was amplified by PCR. Transcripts of the PCR product were used for the next round of selection. The filter binding assay was used to monitor the progress of selection after each SELEX cycle. The binding affinity was evaluated as the percent of the RNA retained on the filter in the total RNA pool. When compared with the starting RNA pool (1-RNA) from which 0.1% of the input RNAs were retained on the membrane, the ninth RNA library (9-RNA) had 9.72% of the input RNA bound. No further enrichment was seen following additional selection rounds (Supplementary Figure S1), suggesting that maximal binding of the RNA pool had been reached. The binding activities of the RNA pools were further confirmed by gel shift assays (data not shown). These results indicated that the RNA pool was successively enriched in ligands with high binding specificity for the target protein.

The highly enriched aptamer pools (12-RNA) were cloned and sequenced. The individual clones were classified into six different groups based on the alignments of individual aptamer sequences (Table 1). About forty percent of the clones (Group I and II aptamers) included a conserved sequence, which is comprised of 13 nucleotides A(A/G)TTGAGGGACC(A/G). No common secondary structural motifs in the six groups were found using secondary structure predictions based upon the RNA folding algorithms Mfold and Quickfold. One representative sequence from each group (A-1, A-5, A-9, A-12, A-28 and B-68) was chosen for further characterization because of their relative abundance within their group. The dissociation constants (K_d) for selected aptamers with the target protein were calculated from a native gel mobility shift assay (Figure 1A). Three of the aptamers showed good binding kinetics to gp120. The apparent K_d values of A-1 and B-68 were about 52 nM and 97 nM (Figure 1B and Supplementary Figure S2), respectively. The filter binding assay (data not shown) also confirmed the binding activity of these individual aptamers. These aptamers also showed selective binding with the HIV-1_{BaL} gp120 (data not shown) *versus* the HIV gp120 CM protein.

For the sake of comparison, a 2'-F modified RNA aptamer B40t77 that is specific for the conserved co-receptor region of the gp120 of the R5 strain of HIV-1 previously developed (28) by the James' lab was also tested in the gel shift assays using the HIV-1_{BaL} gp120 protein. Interestingly, the B40t77 aptamer did not show binding affinity to this target protein (data not shown), suggesting that B40t77 and our selected aptamers recognize different epitopes of the HIV-1_{BaL} gp120 protein. Since the

Table 1. The alignment and identification of RNA aptamers

Groups	RNA	Random sequences	Frequency (140 clones)
I	A-1	<u>AATTGAGGGACCACGCGCTGCTTGTGTGATAAGCAGTTTGTGCGTGATGG</u>	33 (23.6)
	B-7	<u>AATTGAGGGACCAACGCGAGGATGTGGATAGTGTGTA</u> <u>TTTGCCTTGATGG</u>	3
	A-32	<u>AATTGAGGGACCGTTGGTAAAAGCCGGAAAT</u> <u>TGAGCTTTTACGGCGATGG</u>	5
	B-55	<u>AATTGAGGTACCGCGTTATTAGGAACAAAT</u> <u>TGGAATCTAAACGCGATGG</u>	2
	A-24	<u>AATAGAGGGACCCAGATATAGGCTACACGGATGATGGTGTATCTGGATGG</u>	1
	B-19	<u>AATAGAGGAACCGTTTCAGAAGACTACAGGTTAGTCCAATGAAGCGACGG</u>	1
	B-31	<u>AATAGAGGGACCGTGGACAATAATTTATGGTCA</u> TTTATTGGCACCATGG	1
	A-12	<u>AGTAGAGGAACCAAGCAATGGATGAATGCAAAAGTG</u> <u>TAAATGCTTGATGG</u>	10 (7.1)
	II	A-9	<u>TGAGTTTGGGTAATTTCCGGTTTCCGGTTTACTCACGAAAGATCGGTCCG</u>
III	A-28	<u>TAAAGGAGGGAAGGATGAGACCGCACGAAAAATATCAGCATA</u> <u>CGTTTGTG</u>	10 (7.1)
IV	A-5	<u>GAAACTAGTTTGAATAATGGTGTAGAGGAGGGTCAATAGTTT</u> <u>CGTTGGTG</u>	9 (6.4)
V	B-68	<u>ACATAGTAATGACACGGAGATGGAGAAAAAACAGCCATCTCT</u> <u>TGACGGT</u>	2
VI		Orphan sequence	48

Following the 12th round of selection, the selected RNA pool was cloned and sequenced. After alignment of all 140 clones, six groups of anti-gp120 aptamers were identified. Only the random sequences of the aptamer core regions (5'-3') are indicated. The conserved sequences in the group I and II were underlined. Isolates occurring with multiple frequency are specified. Values are given as *n* (%).

HIV-1_{BaL} gp120 proteins used in aptamer development from these studies are from different sources, this may explain their different binding affinities for gp120.

Anti-gp120 aptamer specifically binds and is internalized by cells expressing HIV gp160

CHO-gp160 cells stably expressing the HIV envelope glycoprotein gp160 were used to test for binding and internalization of the selected anti-gp120 aptamers. These cells do not process gp160 into gp120 and gp41 since they lack the *gag* encoded proteases required for envelope processing. As a control we used the parental CHO-EE cell line, which does not express gp160. The anti-gp120 aptamer and the truncated A-1 aptamers were labeled with Cy3 to follow their binding and uptake. Flow cytometric analyses (Figure 2A) revealed that the aptamers specifically bound to the CHO-gp160 cells but did not bind to the control CHO-EE cells.

In order to determine if the bound aptamers were internalized in the gp160 expressing cells, we carried out real-time live-cell Z-axis confocal microscopy with the CHO-gp160 cells incubated with the Cy3-labeled transcripts (Figure 2B and C). After 2 hours of incubation, the Cy3-labeled aptamer was selectively internalized within the CHO-gp160 cells, but not the CHO-EE control cells (Figure 2D). To visualize the nucleus, the cells were stained with the nuclear dye Hoechst 33342 before incubation with the Cy3 labeled-aptamer. Figure 2C showed that the aptamer aggregated within the cytoplasm suggesting that the gp120 aptamers maybe enter cells *via* receptor-mediated endocytosis.

Anti-gp120 aptamers inhibit HIV-1 infection of CEM T-cells

In a previous study, investigators demonstrated that anti-gp120 aptamers provided significant anti-HIV function *via* binding to the viral envelope and preventing viral interaction with the cellular CD4 receptor (28).

We therefore carried out an assay to determine if our anti-gp120 aptamers would also block HIV infectivity in cell culture. In this assay, the aptamers were incubated with HIV-1 infected-CEM cells four days after the cells were challenged with the virus. At different days post-treatment with the aptamers, aliquots of the media were assayed for viral p24 antigen levels (Figure 3). The results showed that the anti-gp120 aptamers (A-1 and B-68) inhibited HIV-1 p24 production and provided somewhat more potent inhibition than the previously published B40t77 (28,43) (designated here as aptamer 1).

Design of anti-gp120 aptamer-siRNA chimera delivery systems that bind and are internalized by cells expressing HIV gp160

We next asked whether or not our newly selected aptamers could be used as delivery vehicles for our anti-HIV siRNA as previously reported (43). The aptamer-siRNA chimeras (**Ch A-1** and **Ch B-68**) (Figure 4A) were generated as previously described (43) and a two nucleotide linker (UU) was inserted between the aptamer (A-1 or B-68) and the Dicer substrate anti-*tat/rev* siRNA portion to increase molecular flexibility.

To evaluate the potency of the siRNA alone, we used a psiCheck reporter system in which the siRNA target was inserted in the 3'-UTR of the Renilla luciferase gene. The silencing of both the sense and antisense orientations of the target were tested and the selectivity ratios were calculated as a measure of the relative knockdown efficiencies for each orientation (Supplementary Figure S3). The chimeras **Ch A-1** and **Ch B-68**, respectively mediated ~80% and 75% knockdown of the sense target; however, knockdown of the anti-sense target was much less (20–40%), indicative of good strand selectivity ($R_{Ch A-1} = 3.3$; $R_{Ch B-68} = 3.4$), which is comparable with **Ch L-1** (43).

Next, the binding affinities of the chimeras for gp120 were assessed by using a gel shift assay (Figure 4B) and

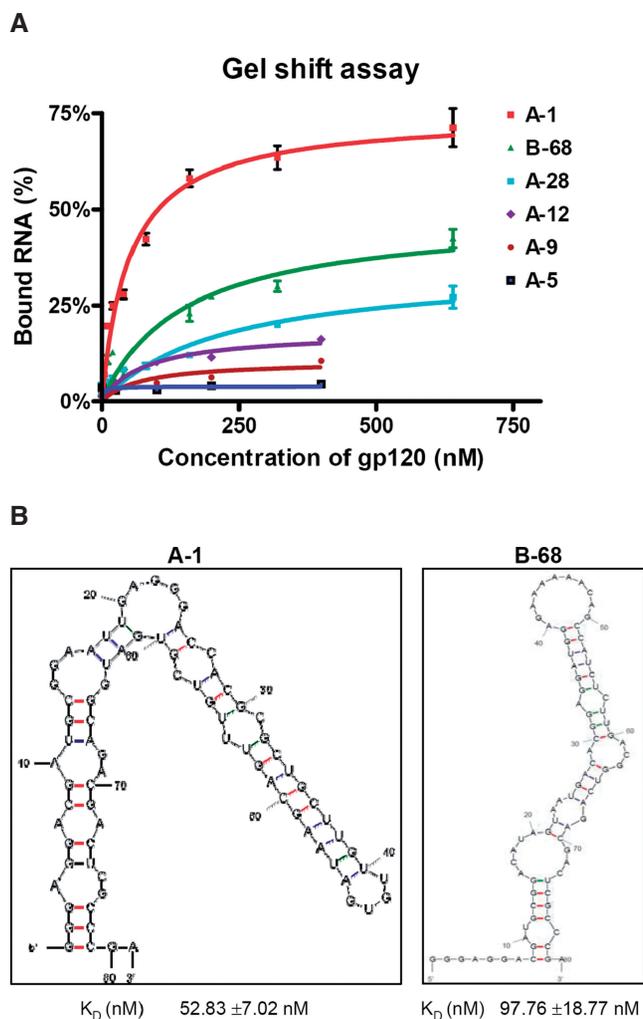


Figure 1. Binding activity assay and predicted secondary structure of selected individual aptamers against HIV-1_{BaL} gp120. (A) Binding curve from a gel shift assay. The 5'-end P³²-labeled individual aptamers were incubated with the increasing amounts of gp120 protein. The binding reaction mixtures were analyzed by a gel mobility shift assay. Aptamer A-1 and B-68 showed the best binding affinity with the target protein. (B) The predicted secondary structures of the anti-gp120 aptamer A-1 and B-68 as predicted by QuickFOLD and the calculated K_D determinations are indicated. Data represent the average of four replicates.

flow cytometry (Figure 4C). These data indicate that the chimeras maintain approximately the same binding affinities as the aptamers alone. To determine if the bound chimeras were internalized in the gp160 expressing cells, we carried out Z-axis confocal microscopy with the CHO-gp160 cells incubated with Cy3-labeled transcripts. The time-course images (Supplementary Figure S4) showed that Cy3-labeled chimera **Ch A-1** was successfully internalized into the cytoplasm of cells. To visualize the nucleus, the cells were stained with the nuclear dye Hoechst 33342 before incubation with Cy3-RNA. As expected, no uptake of the chimera was observed with the CHO-EE control cells (data not presented).

Anti-gp120 aptamer-siRNA chimeras inhibit HIV-1 infection of CEM T-cells and human PBMCs

To verify the anti-HIV activity of the chimeras in inhibiting HIV-1 replication, we carried out the HIV-1 challenge assays as previously described (43). In this assay, the experimental RNAs were incubated with HIV-1 IIIB infected-CEM cells or HIV-1 NL4-3 infected human PBMCs. The results of p24 antigen analyses (Figure 5A and B) showed that each of the aptamers and chimeras inhibited p24 production, but the strongest inhibition was observed with the chimera **Ch A-1** treatment. In particular, A-1 and **Ch A-1** showed stronger suppression of HIV-1 than aptamer 1 and **Ch L-1** previous reported (43) at 7 and 9 days of posttreatment in PBMCs.

To confirm that the siRNA component was functioning along with the aptamer, following internalization of the **Ch A-1** chimera in infected cells, we also evaluated the relative levels of inhibition of *tat/rev* gene expression. Aptamers or chimeras were added directly to media containing infected human PBMCs. After 7 days of post-treatment, treated cells were harvested, the total RNA was extracted and the expression level of *tat/rev* mRNA was determined by quantitative RT-PCR expression assays. We find that the treatment of infected cells with the chimeras is able to induce silencing of the *tat/rev* gene, while the aptamer alone did not affect *tat/rev* gene expression (Figure 5C). These results provide further support that the aptamer delivered siRNA triggers RNAi.

Design of anti-gp120 aptamer-stick-siRNA delivery systems that bind and are internalized by cells expressing HIV gp160

An ideal aptamer chimera for use against HIV-1 would have the capacity for easily combining different siRNAs to avert the viral escape mutants. A new delivery system design based on the A-1 aptamer was constructed which facilitates the ready exchange of siRNAs associated with the aptamer (Figure 6A). In this design format the aptamer and siRNAs are linked *via* 'stick' sequences consisting of 16 nt at the aptamer 3'-end which are complementary to 16 bases on one of the two siRNA strands. After a simple incubation of the aptamer and the pre-formed siRNA with this 'sticky' sequence, a stable base-paired chimera is formed (Figure 6A and Supplementary Figure S5). In the present design, all the RNA components are chemically synthesized. The siRNA portion targets the HIV-1 *tat/rev* common exon sequence. Seven three-carbon atoms (C3) as linker was put between RNA strand and stick sequence in order to provide molecular flexibility, not interfering aptamer correct folding. Aptamer A-1 with 'sticky' end (**A-1-stick**) and sense strand of siRNA were 2'-fluoropyridinium modified, which enhance their RNase resistance in mouse serum (data not shown).

The psiCheck reporter system was used to evaluate the RNAi potencies and strand selectivity of the

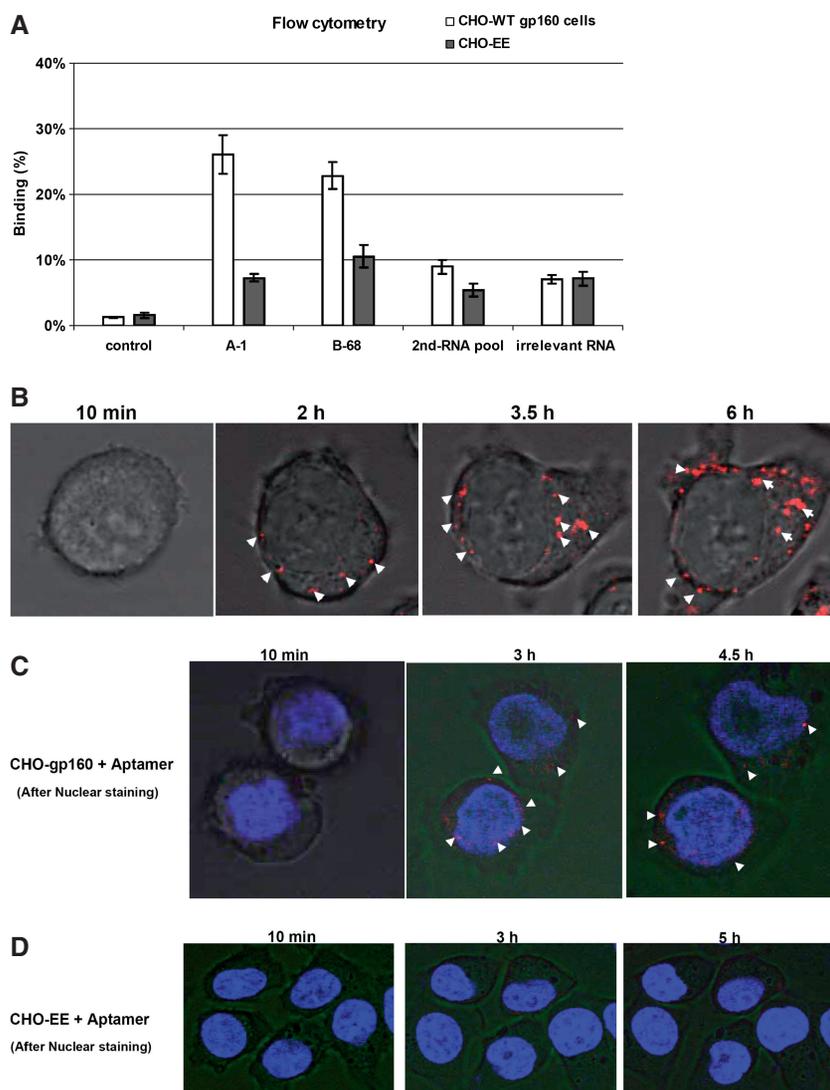


Figure 2. Cell-type specific binding and uptake studies of aptamers. (A) Cell surface binding of Cy3-labeled RNAs was assessed by flow cytometry. Cy3-labeled RNAs were tested for binding to CHO-gp160 cells and CHO-EE control cells. The selected aptamers showed cell-type specific binding affinity. The 2nd RNA pool and irrelevant RNAs were used as negative controls. Data represent the average of three replicates. (B) Internalization analysis. CHO-gp160 cells were grown in 35 mm plates and incubated with a 100 nM concentration of Cy3-labeled A-1 in culture media for real-time live-cell-confocal microscopy analysis. The images were collected at 15 min intervals using 40 \times magnification. (C,D) Localization analysis. CHO-gp160 cells (C) and CHO-EE control cells (D) were grown in 35 mm plates. Before incubation with 100 nM of Cy3-labeled A-1, cells were stained with Hoechst 33342 (nuclear dye for live cells) and then analyzed using real-time confocal microscopy.

aptamer-stick-siRNA system. The gene silencing of both the sense (corresponding to the mRNA) and the anti-sense strands were tested independently (Supplementary Figure S6). As expected, N-1 and N-2 down-regulated the sense target expression and had a good strand selectivity ($R_{N-1} = 4.0$; $R_{N-2} = 2.1$). From these results we conclude that the siRNA component of the 'stick' aptamer-siRNA chimeras mediated the desired target knock-down and exhibits a strand bias in favor of the desired anti-*tat/rev* guide sequence. Interestingly, the N-1 design mediated more efficient knockdown of the sense target

(~85%) than the N-2 design (~55%), suggesting that more potent Dicer-cleavage might be generated from the N-1 design.

We also carried out gel mobility shift assays to monitor the gp120 binding capabilities of these chemically synthesized chimeras and observed binding to the target protein with nanomolar dissociation constants (Figure 6B). These synthetic 'stick' chimeras selectively bind to cells expressing gp160 (Figure 6C) and are internalized into the cytoplasm (Figure 6D). The live-cell confocal images demonstrated that N-1 containing a

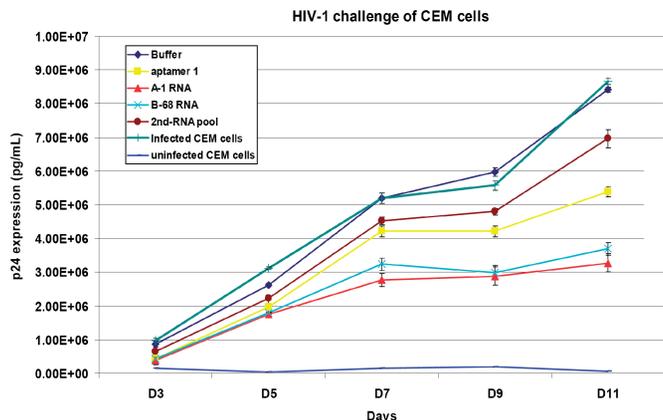


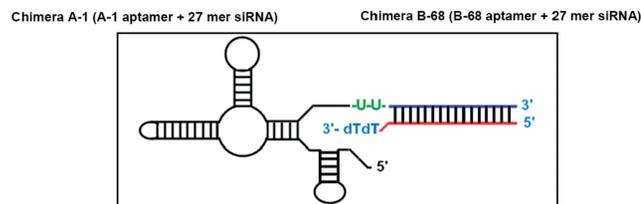
Figure 3. The selected anti-gp120 aptamers inhibit HIV-1 replication in cells previously infected with HIV-1. The 1.5×10^4 HIV-1 infected CEM cells and 3.5×10^4 uninfected CEM cells were incubated at 37°C with the various experimental RNAs at a final concentration of 400 nM . The culture supernatants were collected at different time points following addition of the aptamers (3 d, 5 d, 7 d, 9 d and 11 d) for p24 antigen analyses. Data represent the average of triplicate measurements of p24.

5'-end Cy3-labeled sense strand successfully delivers siRNA into the cytoplasm of gp160 expressing cells.

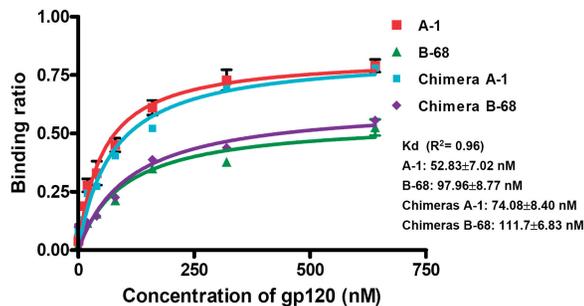
Aptamer-based delivery systems are processed by Dicer

To determine whether or not human Dicer can process the siRNA portion of these chimeras, either the sense or antisense strands were end-labeled with $\gamma\text{-}^{32}\text{P}\text{-ATP}$ and subsequently used in formation of the chimeras (Figure 7A). The chimeras were incubated with recombinant human Dicer and the cleavage products were analyzed by denaturing gel electrophoresis. The size of the ^{32}P labeled cleavage product(s) indicates from which direction Dicer enters the siRNA and cleaves (Figure 7A and Supplementary Figure S7). When chimera **Ch A-1** was incubated with the human Dicer and the ^{32}P -label was placed on the 5'-end of the antisense strand (Figure 7A and B), we observed that the primary processing takes place *via* Dicer entry from the opposite side of the aptamer since the majority of the ^{32}P -labeled resides in the 21- to 23-mer sized product as opposed to the shorter 6nt product, which derives from Dicer entry on the aptamer side of the duplex. For the **N-1** and **N-2** 'stick' aptamer chimeras, Dicer only processes these from side containing the 2 base 3' overhang since only a single species of ^{32}P -labeled product is seen regardless of whether the label is at the 5'-end of the sense or antisense strand (Figure 7B). Since the positions of the sense and antisense strands differ between **N-1** and **N-2** with respect to the two base 3' overhang, Dicer cleavage results in predictably different 21–23 siRNA products from these two configurations (Supplementary Figure S7) which can have different potencies.

A Design of aptamer-siRNA chimeras



B Gel shift assay



C Flow cytometry

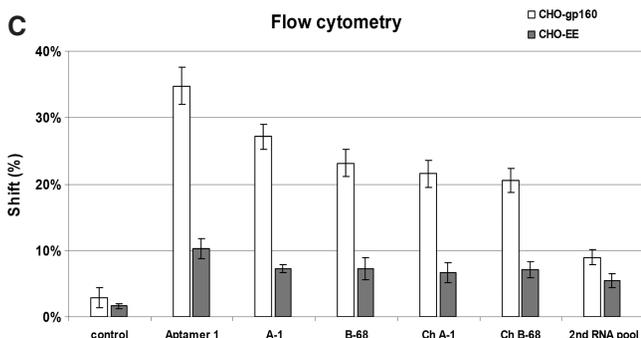


Figure 4. The design and binding activity of the aptamer-siRNA delivery system. (A) Schematic aptamer-siRNA chimeric RNAs: the region of the anti-gp120 aptamer is responsible for binding to gp120 and the siRNA is targeting a common exon of HIV-1 *tat/rev*. A linker (UU) between the aptamer and siRNA is indicated in green. (B) The aptamer-siRNA chimeric RNAs that have comparable K_d values as well as parental aptamers specifically bind the HIV_{BaL} gp120 protein. Data represent the average of three replicates. (C) Cell-type specific binding studies of aptamers. Cy3-labeled RNAs were tested for binding to CHO-gp160 cells and CHO-EE control cells. Cell surface bindings of Cy3-labeled RNAs were assessed by flow cytometry. The selected aptamers showed cell-type specific binding affinity. The 2nd RNA pool and irrelevant RNA were used as negative controls. Data represent the average of two replicates.

Anti-gp120 aptamer-stick-siRNA system inhibits HIV-1 infection in primary human PBMCs

In order to test the anti-HIV activity of the aptamer-stick-siRNAs for their inhibition of HIV-1 replication, we carried out HIV-1 challenge assays as described above. The experimental RNAs were incubated with HIV-1 Bal infected human PBMCs and over a period of several days the HIV encoded p24 antigen levels were measured (Figure 8A). These results verified that each of the

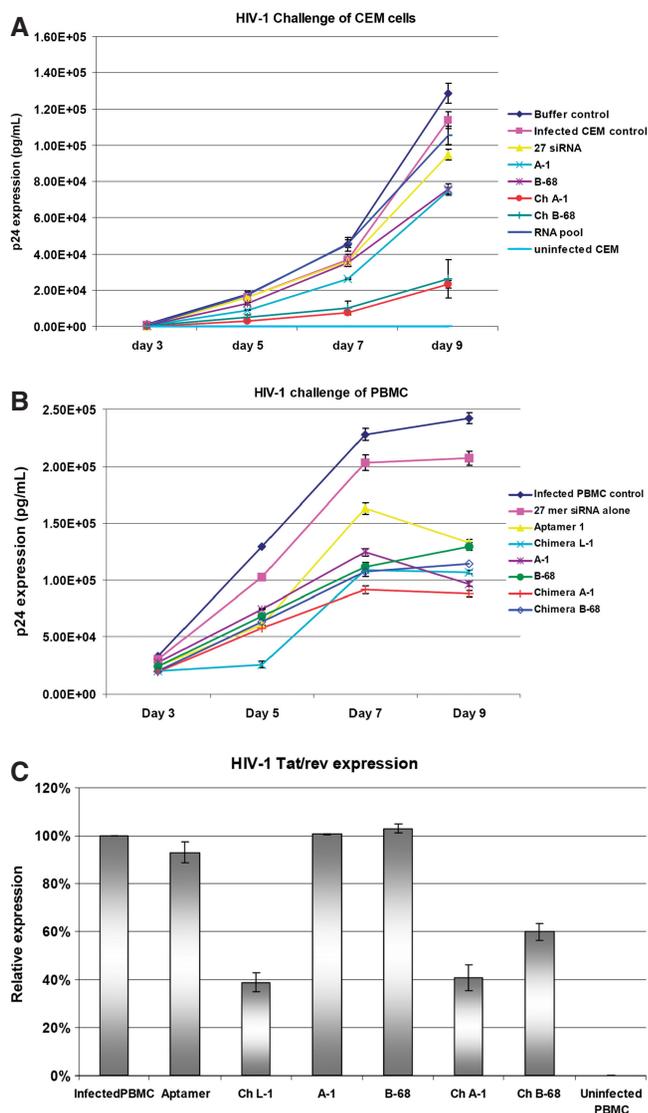


Figure 5. Dual inhibition of HIV-1 infection mediated by aptamer-siRNA chimeras. Both anti-gp120 aptamer and aptamer-siRNA chimeras neutralized HIV-1 infection in (A) CEM cells (IIIB strain) and (B) human PBMCs (NL4-3 strain) culture, respectively. Data represent the average of triplicate measurements of p24. The chimeras (Ch A-1 and Ch B-68) showed stronger inhibition than aptamer alone indicating that (C) the siRNA delivered by the aptamers down-regulated *Tat/rev* gene expression in the PBMCs. Data represent the average of three replicates.

aptamer and aptamer-stick-siRNA combinations inhibited p24 production. As we have previously observed, the best suppression of HIV replication derived from the chimeric aptamer-siRNAs Ch A-1 and N-1. We also carried out qRT-PCR to evaluate the down-regulation of *tat/rev* gene expression to confirm the siRNA function following internalization within infected PBMCs. After 7 days of treatment, treated cells were harvested, the total RNA was extracted and the extent of *tat/rev* gene inhibition was determined by quantitative RT-PCR.

The N-1 chimera inhibited *tat/rev* expression by ~50% relative to the controls, whereas the aptamer alone did not affect the levels of *tat/rev* gene expression (Figure 8B). For comparison, N-2 gave somewhat less target knockdown than the N-1 and Ch A-1 chimeras.

An ideal chimeric aptamer-siRNA delivery system targeting HIV would have the capacity for combining various siRNAs to avert viral escape mutants. HIV-1 exploits multiple host proteins during infection. It has been demonstrated that more than 250 HIV-dependency factors (HDFs) are required by HIV infection (48). These proteins participate in a broad array of cellular functions and implicate new pathways in the viral life cycle. Therefore, we took advantage of the 'stick' bridge to tether the anti-gp120 aptamer with different siRNA portions targeting HIV-1 dependency factors. The examples shown (Figure 9) include TNPO3 (known as transportin 3, a karyopherin required for viral integration) and CD4 (the primary receptor for viral entry).

The HIV-1 challenge assays were performed as described above. The experimental RNAs were incubated with HIV-1 IIIB infected CEM cells and the HIV encoded p24 antigen levels were measured (Figure 9A). In all cases, the aptamer-stick-siRNA combinations (*Tat/rev* N-1, TNPO3 N-1, CD4 N-2 and the siRNA-cocktails) provided more potent inhibition than the aptamer alone. Knockdown of target gene expression was further evaluated by qRT-PCR, which confirmed for each combination that the siRNAs were functioning following internalization in the HIV infected CEM cells. The CD4 N-2 chimera inhibited CD4 expression by ~75% relative to the controls, whereas the aptamer alone did not affect the levels of CD4 gene expression (Figure 9B). The *tat/rev* N-1 and TNPO3 N-1 also gave ~50% silencing for the corresponding target genes. For comparison, the three siRNA-cocktails gave somewhat less individual target knockdown than the single siRNA chimera, suggesting that there may be competition for RISC loading between the various siRNAs. We have not yet optimized the amounts of each individual chimera in the mix required to minimize this type of competition.

The aptamer-based delivery system does not trigger a type I interferon response

It has been reported previously that siRNAs delivered by liposomes or polyplex reagents can non-specifically activate inflammatory cytokine production (TNF α , IL-6 and IL-12) as well as IFN responsive genes, which in turn can trigger cellular toxicity. We therefore assessed the induction of type I interferon regulated gene expression by our anti-gp120 aptamer-based siRNA delivery system using quantitative RT-PCR expression assays. We used IFN- α as a positive control to confirm up regulation of p56 (CDKL2) and OAS1 gene expression. The treatment of HIV-1 infected CEM cells (data not shown) with the chimeras did not induce type I IFN responses. Similar results were obtained using HIV infected primary human PBMCs treated with these chimeric aptamer-siRNAs

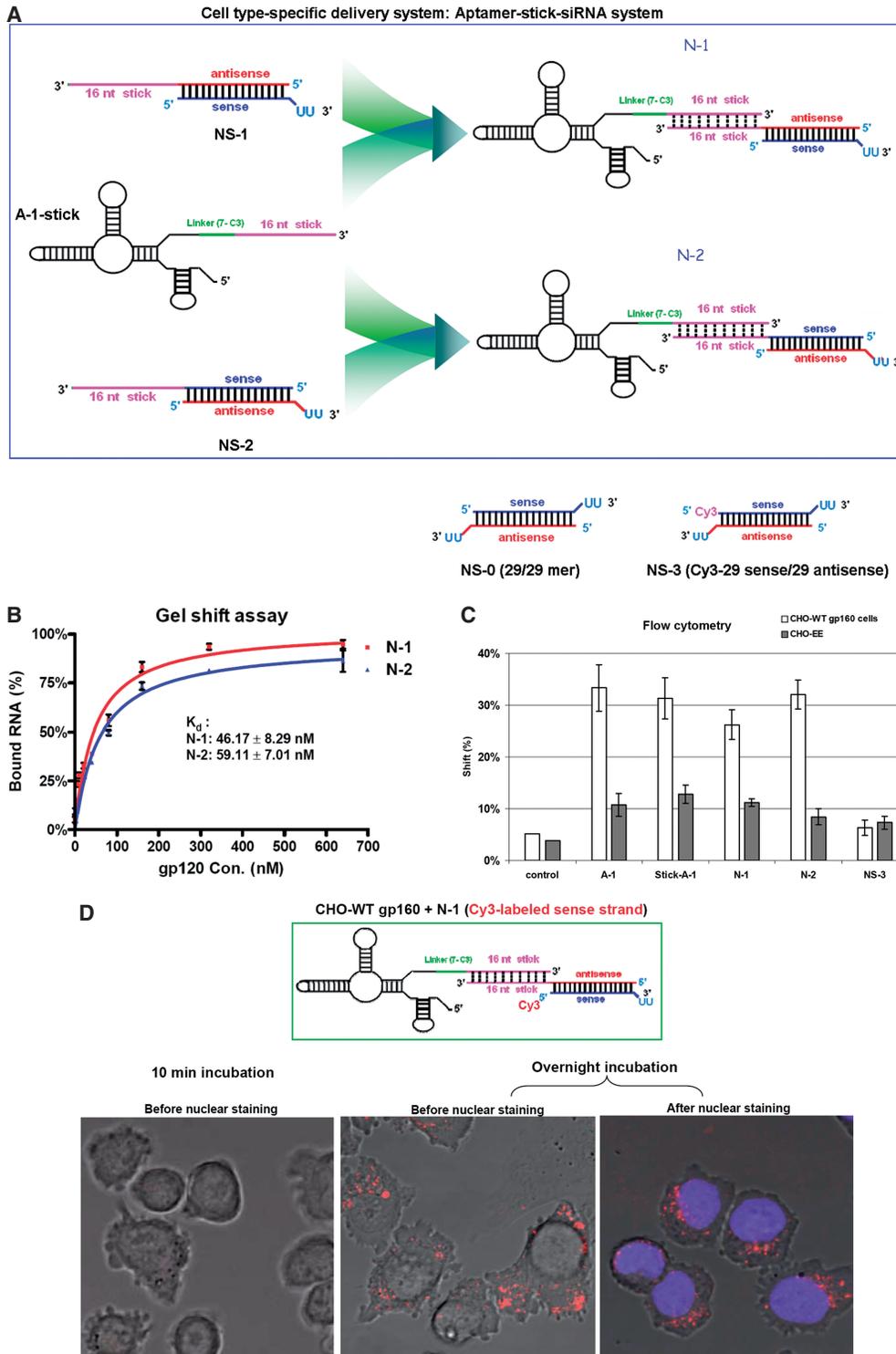


Figure 6. Cell-type specific aptamer-stick-siRNA delivery system. (A) Schematic aptamer-stick-siRNA system (N-1 and N-2). The anti-gp120 aptamer A-1 and the 27mer siRNA targeting HIV-1 *tat/rev* are shown. The siRNA is linked to the aptamer portion *via* the “stick” sequence which consists of 16 nt appended to the aptamer 3'-end allowing complementary interaction of one of the two siRNA strands with the aptamer. After a simple incubation of the A-1-stick and NS-1 or NS-2 siRNA configurations, they form stable base-pairing. A linker of seven three-carbons between the aptamer RNA and the stick portion is used to avoid steric interaction of the stick with the aptamer. (B) The aptamer-stick-siRNA system (N-1 and N-2) has comparable K_d values to the aptamer A-1 alone in binding assays with the HIV_{Bal} gp120 protein. Data represent the average of two replicates. (C) Cell-type specific binding studies of aptamer-stick-siRNA. Cy3-labeled RNAs were tested for binding to CHO-gp160 cells and CHO-EE control cells. Binding of the Cy3-labeled RNAs was assessed by flow cytometry. The selected aptamers show cell-type specific binding affinities. A 27 mer duplex RNA (NS-3) with a 5'-Cy3-labelled sense strand is used as a negative control. Data represent the average of two replicates. (D) Internalization and intracellular localization analyses. CHO-gp160 cells were grown in 35 mm plates and incubated in culture medium with a 100 nM concentration of N-1 containing a 5'-Cy3-labeled sense strand for real-time live-cell confocal microscopy analysis as previously described. After overnight incubation, cells were stained with Hoechst 33342 (nuclear dye for live cells) and then analyzed by confocal microscopy.

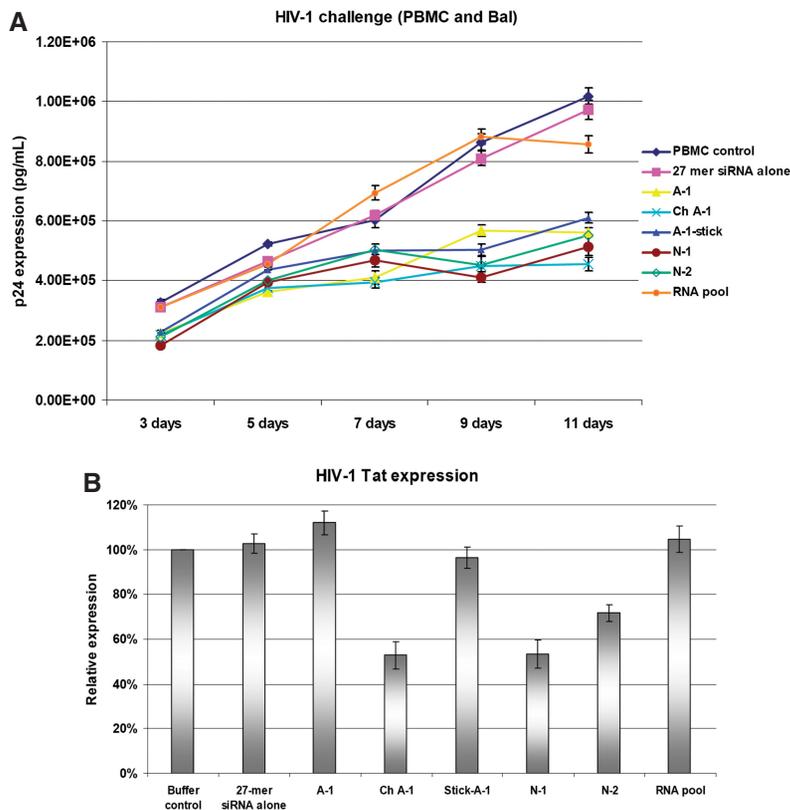


Figure 8. Dual inhibition on HIV-1 infection mediated by aptamer-based siRNA delivery system. (A) Both anti-gp120 aptamer, aptamer-siRNA chimeras (Ch A-1) and aptamer-stick-siRNA (N-1 and N-2) neutralize HIV-1 Bal infection in PBMCs. The chimeras Ch A-1, N-1 and N-2 were more potent inhibitors than the respective aptamers alone. Data represent the average of triplicate measurements of p24. (B) The siRNA delivered by aptamers down-regulates *tat/rev* gene expression in human PBMCs. Data represent the average of three replicates.

(Supplementary Figure S8), suggesting that the gp120 mediated internalization of the chimeras does not trigger toxic IFN responses.

DISCUSSION

Nucleic acid aptamers with binding affinities in the low to mid-nanomolar range have been utilized for flexible applications ranging from diagnostic to therapeutic assay formats. Moreover, aptamers that target specific cell surface proteins are employed as delivery molecules to target a distinct cell type, hence reducing off-target effects or other unwanted side effects. We have capitalized on the exquisite specificity of a gp120 aptamer to deliver anti-HIV siRNAs into HIV infected cells with the net result that the replication and spread of HIV is strongly inhibited by the combined action of the aptamer and a siRNA targeting the *tat/rev* common exon of HIV-1. Therefore, the anti-gp120 aptamers can function both as antiviral agents and as siRNA delivery reagents.

It is highly desirable to generate new aptamers to expand their diversity for *in vivo* applications. In a therapeutic setting, anti-gp120 aptamers and various siRNAs targeting different genes may need to be interchanged to

avert viral resistance. In the present work, a series of new 2'-F substituted RNA aptamers that specifically bind to the HIV-1_{BaL} gp120 protein with nanomolar affinity were successfully isolated from an 81 nt RNA library *via* SELEX (Systematic Evolution of Ligands by EXponential enrichment). The selected aptamers are able to specifically bind gp120 and are internalized into cells expressing the HIV envelope. We observe an aggregation of aptamer within the cytoplasm as revealed by confocal microscopy, suggesting that the aptamers are internalized by gp120-mediated endocytosis. In addition, these aptamers have been shown to inhibit several different HIV-1 strains, indicating that anti-gp120 aptamers that inhibit the initial step of HIV-1 entry by blocking the binding of the gp120 and CD4 receptor may be useful in HIV-1 therapeutic applications. Compared with the previously published B40t77 aptamer that was selected against the HIV-1_{BaL} gp120 protein through BIAcore biosensor chip-based SELEX, our selected aptamers show somewhat better inhibitory activity. The fact that B40t77 did not bind with our target protein suggests that the different sources of target and selection methods can affect the aptamer-target protein binding epitopes.

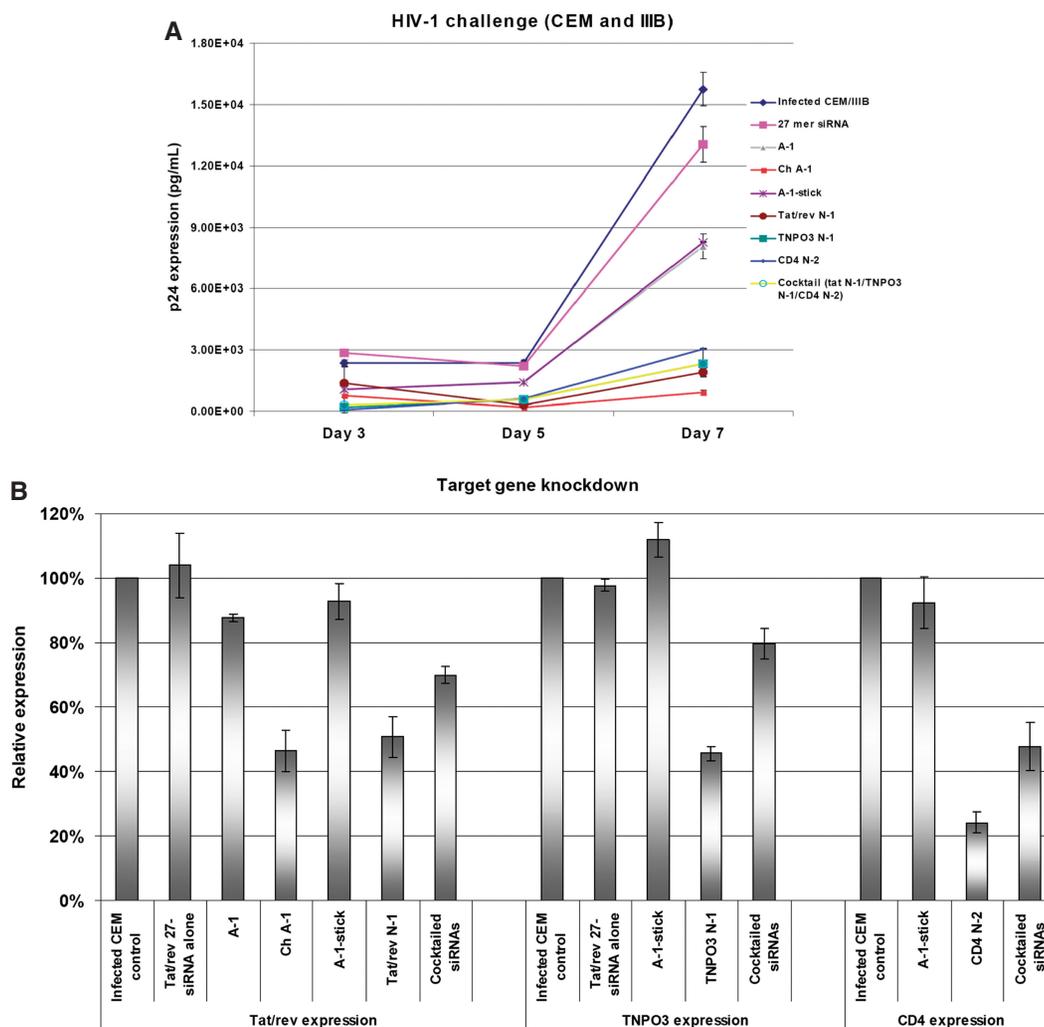


Figure 9. The HIV-1 inhibition mediated by the various siRNAs delivered using the 'stick' aptamer system. (A) Three different siRNAs which target the HIV-1 *Tat/rev*, human CD4 receptor and HIV-1 dependency factor TNPO3 RNAs, were annealed to aptamer A-1 *via* the 'stick' bridge. The resulting aptamer-stick-siRNAs (*Tat/rev* N-1, TNPO3 N-1 and CD4 N-2) neutralized HIV-1 infection in CEM cell culture, providing more potent inhibition than the aptamer alone. The combination of three siRNAs also suppressed the HIV-1 replication. Data represent the average of triplicate measurements of p24. (B) The siRNAs delivered by aptamers down-regulates target gene expression in the HIV infected CEM cells (HIV-1 *tat/rev*, TNPO3 and CD4). Data represent the average of three replicates.

On the basis of our previous results, two kinds of dual inhibitory functional anti-gp120 aptamer-based delivery systems were constructed and evaluated for HIV-1 inhibition. In the first design, we cotranscribed the aptamer-siRNA sense single strand, followed by annealing of the complementary siRNA antisense strand to complete the chimeric molecule. Specifically, the secondary design is a worthy point of discussion. A new anti-gp120 aptamer-stick-siRNA delivery system has been designed which allows mixing new siRNAs with the same aptamer, which could ultimately be useful for minimizing viral escape mutants. Both the aptamer and siRNA portions are chemically synthesized and subsequently annealed *via* a complementary 16-nt 'stick' sequence which forms a stable base-pair *via* the 'sticky' bridge. The linker, seven sets of three-carbon atoms (C3), between the RNA and

the stick end provides molecular flexibility and minimizes steric hindrance which could impede annealing of the two RNAs. These design strategies not only guarantee tight conjugation of the aptamer with the siRNA portion, but also do not interfere with correct folding of the aptamer and the Dicer processing of the siRNA. Most importantly, since this kind of design is quickly assembled from two chemically synthesized RNA portions, each portion is easily changed to avert viral resistance. So far, the chemical synthesis of long RNAs is limited by the length and backbone modifications of the RNAs. Using the 'sticky bridge' strategy, we have been able to chemically synthesize a 2'-fluoropyrimidine modified aptamer (81 nt plus 16-base bridge) separately from the 27 bp (plus 16 base bridge) siRNA in milligram scale, followed by *in vitro* joining of the two RNAs. This attractive 'sticky'

bridge-based approach can potentially be used for mixing different siRNAs with a single aptamer to multiplex target down regulation, and in the case of HIV infection, to avert resistance to the siRNA component.

We demonstrate that our delivery systems specifically bind to the surface of cells expressing gp160 and are internalized, allowing functional processing of the siRNA into RISC, resulting in specific inhibition of HIV-1 replication and infectivity in cell culture. Both of the anti-gp120-based siRNA delivery systems serve as dual function inhibitors and therefore provide greater efficacy than either the aptamer or siRNA applied alone.

Interestingly, even though our designs (**Ch A-1**, **N-1** and **N-2**) are able to down-regulate the target expression and show good strand selectivity, their RNAi potencies are different. Previous studies of Dicer substrate siRNAs (49,50) used substrates in which Dicer entry on the blunt end of the DsiRNAs was completely blocked by placing two deoxy nucleotides at the 3'-end of the desired passenger strand, creating an absolute polarity for Dicer entry from the two base 3' overhang. The present studies did not employ deoxys so entry from the blunt end of the **Ch A-1** siRNA takes place relatively efficiently relative to the entry from the 2 base 3' overhang, which is adjacent to the aptamer that creates a steric hindrance for Dicer entry (Figure 7B). Also, in contrast to our previous observations, (49,50) the preference for guide strand selection in our chimeras does not necessarily favour the strand with the two base 3' overhang (Supplementary Figures S6 and S7), since the more potent configuration **N-1** has the guide strand positioned with the 5'-end at the side of Dicer entry. In this context it should be noted that the strand selectivity of **N-1** is not as good as that of **N-2** (Supplementary Figure S6). The difference in potency between **N-1** and **N-2** most likely resides in the species of 21- to 23-mer produced, which differ between the two forms. Interestingly, Dicer does not process the 'stick' aptamer siRNAs at all from the blunt end side as it does with the Ch A-1. We attribute this to the lack of a free 3'OH in these chimeras, in which the end of the siRNA is covalently attached to the 'stick' sequence which in turn forms a 2'OMe modified duplex with the aptamer. The 2'OMe backbone modifications throughout the 'stick' also block Dicer cleavage (51). Since the strand selectivity of Dicer generally favors the strand with the 2 base 3' overhang (49,50), it is important to design the Dicer substrates such that a potent siRNA against the desired target generated from the cleavage event. This will minimize the off target potential of the passenger strand.

In summary, we have demonstrated that the anti-gp120 aptamers not only provide a potential new drug therapy approach for combating HIV infection, but also act as delivery vehicles for siRNAs and perhaps other small RNA inhibitors. The 'sticky' bridge-base approach offers a major advantage in both chemical synthesis and the opportunity to mix and match the aptamer with different siRNAs in a non-covalent fashion. For HIV clinical

applications, the mixing approach may be a requirement for averting viral escape mutants.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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